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"Encapsulation and fluidisation maintains the viability and glucose sensitivity of beta-cells"

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Abstract

This study presents experimental data of a fluidised-bed bioreactor for the cultivation of encapsulated pancreatic beta-cells. The fluidisation quality for the bioreactor was evaluated at different flow rate using bed-expansion parameters. Homogeneous distribution of microcapsules was achieved at a flow rate of 2000 µL/min. This enabled efficient contact between the encapsulated cells and medium, which contributed to high cell viability. Microcapsule breakage was < 4% on day 7 and confirmed the stability of encapsulated systems under fluidised culture. Importantly, endocrine beta-cells cultured in the bioreactor were shown to be dramatically more responsive to changes in glucose concentration compared to static culture ($P < 0.001$). Based on these results, cultivation of encapsulated cells in a fluidised bioreactor, especially for pancreatic beta-cells that are limited in supply, is a promising approach to address the lack of a safe method for storage and handling of cells between laboratories and clinical sites prior to transplantation.

Keywords: microcapsules, endocrine beta-cells, bioreactor, viability, fluidisation, insulin secretion.

1 Introduction

During the last two decades, major interest has been developed in the application of microencapsulation technology for cell-based therapies[1]. Encapsulated systems can provide an immunoisolated environment for transplantation of cells. This is critical in the treatment of autoimmune disorders, such as type I diabetes[2]. Currently, an injection of insulin 2-5 times per day is the most common treatment for patients suffering from type I diabetes[3, 4]. Although the advent of

exogenous insulin has improved the life expectancy of diabetics, maintaining normal blood glucose level remains a considerable challenge, and even with frequent monitoring of blood glucose levels, it is difficult to maintain normoglycaemia. Frequent incidents of hypoglycaemia (low blood glucose level) and hyperglycaemia (high blood glucose level) in these patients can lead to complications later in life, including blindness, and kidney disease [5, 6]. Therefore, a promising alternative to achieve minute-to-minute blood glucose regulation is the encapsulation of pancreatic insulin secreting beta-cells in a semi-permeable polymeric membrane, which could allow bi-directional diffusion of oxygen, nutrients, insulin, and other cell products as well as providing an immunoisolated environment for the protection of pancreatic beta-cells from cytokines and T-cells [7, 8].

This concept dates back to the work of Lim and Sun in the 1980s, who encapsulated pancreatic beta-cells within alginate capsules [9]. Since then, important advances within this field have been made through microstructural manipulations of this polymer leading to a larger surface area to volume ratio, increased oxygen and nutrient diffusion rate, and access to more implantation sites by injection [2, 10]. Therefore, a manufacturing method that uses mild conditions to minimise cell damage and a fast production rate, such as electrostatic bead generation and vibrational technology, would be beneficial in cell encapsulation methodology [11-14].

Despite several studies on cell transplantation via microencapsulating technology, their clinical application has been hindered by several issues, including insufficient transfer of nutrients [15] and lack of a storage method for cultivation of encapsulated cells prior to transplantation [1]. A promising methodology to address the issue with storage and distribution of a physiological amount of encapsulated cells between laboratory and clinical site could be the application of bioreactors [1]. Bioreactors not only provide clinical accessibility, but are also associated with a lower risk of contamination compared with manual flask-based culture methods. Economically, these devices are regarded as a more cost effective approach for cell-based therapeutic studies compared to conventional cell culture flasks, owing to less frequent media changes [1, 16, 17].

Stirred tank bioreactors (STR) [18], pneumatically agitated bioreactors (such as bubble column, and air-lift column) [18], membrane bioreactors (such as hollow fibres) [19], fixed-bed bioreactors [17], and fluidised-bed bioreactors [20] are the most typical reactors that have been used for encapsulation of both

plant and mammalian cell culture. Of these systems, hollow fibres are the most studied bioreactors for the cultivation of mammalian cells, which have primarily been used clinically to treat liver failure as a bioartificial liver assist device [21]. The mechanism of hollow fibre bioreactors is based on the cultivation of cells on capillary spaces, and then the flow of fluid or blood can be achieved through the hollow pores [19]. The use of hollow fibre reactors for encapsulated cells has been limited because of a lack of sufficient mass transport between the fibre wall and cells [21]. However, cultivation of cells within fluidised-bed bioreactors provide direct contact between encapsulated cells and the culture medium or physiological fluids, which allow higher mass transfer and biological response *in vitro* [22, 23]. In these bioreactor systems, the difference between the density of encapsulated particles and medium enables the suspension of particles with an upward flow of the medium and hence encapsulated cells float throughout the column without the need for aeration or mechanical agitation [20, 21].

In this study, a vibrating nozzle system was utilised to obtain alginate microcapsules with uniform size and shape. The feasibility of applying a fluidised-bed bioreactor design for the cultivation of pancreatic insulin secreting beta-cells (MIN-6) encapsulated in these uniform alginate microcapsules was then evaluated. The performance of the fluidised-bed bioreactor was studied by evaluating beta-cell viability, insulin secretion ability, microcapsule breakage, and swelling degree. Results from the bioreactor system were compared to that of conventional cell culture flasks.

2 Experimental section

2.1 Cell culture

MIN-6 cells (AddexBio, San Diego, USA) were cultured in Dulbecco's modified eagle medium (DMEM, 4500 mg/L glucose, Sigma, UK) supplemented with 15% fetal bovine serum (FBS, Sigma, UK), 2 mM L-glutamine (Sigma, UK) and 0.05 mM 2-mercaptoethanol (Sigma, UK) [24]. Since mouse pancreatic beta-cells have limited ability to tolerate oxidative stresses, 2-mercaptoethanol was added to the cell culture medium to aid in maintaining a reducing environment; limiting toxic oxygen radicals. All cultures were incubated at 37℃ with 5% CO_2 until they reached 70-80% confluency.

2.2 Preparation of encapsulation solutions

Medium viscosity alginate (≥ 2,000 cP, M/G ratio= 1.56) from brown algae (Sigma, UK) was employed to manufacture microcapsules. For all experiments, alginate was dissolved in sterile deionised water to a concentration of 1%w/v. The cross-linking solution was prepared by dissolving granular calcium chloride (Sigma, UK) in deionised water (100 mM). All solutions were sterile filtered using 0.22 μm syringe filter (Millipore, USA) and were UV sterilised overnight.

2.3 Cell encapsulation

MIN-6 cells were grown in T-75 flasks to ≥80% confluency prior to harvesting. The cells were then detached from the culture flask using Trypsin-EDTA solution (Sigma, UK). After being washed in phosphate buffered saline (PBS) (Fisher scientific), 2 x 10^6 cells/mL were suspended in sterile alginate solution (10 mL). The formation of microcapsules was carried out with the vibrational nozzle technology (A B-395 pro, Buchi, UK), which allowed sterile working condition by providing an autoclavable glass vessel. Formed microparticles were then incubated in a receiving cross-linking solution (100 mM $CaCl_2$) at a distance of 20 cm from the nozzle head for 10 min (Figure 1a). After gelation, the capsules were washed twice with PBS and DMEM to remove any remaining calcium ions from the encapsulated particles. For the conventional extrusion method (control), the microcapsules were produced by the dripping of alginate droplets from a syringe with a needle size of 21 gauge into the cross-linking medium located at 20 cm from the needle tip.

The size distribution of alginate microcapsules produced utilising vibrational technology versus conventional extrusion method was compared by randomly picking 110 microcapsules from each group. Microcapsules were captured with a light microscope (Olympus Co., Germany), followed by measuring the average diameter of particles using image J software (NIH, Bethesda, MD, USA).

2.4 Fluidised-bed bioreactor

The fluidised-bed bioreactor consists of a cylindrical vessel, and a feeding vessel containing 20 mL of the culture medium. A bioreactor with glass column (provided by Dr. Ellis at the University of Bath) with the following dimensions was used in this study: cylinder height=100 mm (with attached bioreactor column heading=80 mm), cylinder diameter= 15 mm, total volume= ~ 15 mL (after

addition of both up and bottom headings). To prevent alginate microcapsules escaping the column, PTFE frits with 100 µm pore size (Kinesis, UK) were added to the bottom and top column headings. While the encapsulated cells were retained in the bioreactor column, the culture medium was pumped axially by a peristaltic pump (Watson-Marlow, UK) through the cylindrical vessel via a circulating loop (Figure 3a). The bioreactor set up was then incubated at 37℃ with 5% CO_2. All bioreactors sections, including glass column, silicone tubing, and feeding vessel were autoclaved at 121°C for 30 min before experimental setup.

2.5 Permeability of alginate capsules to mouse antibodies

To investigate the permeability of the 1%w/v alginate microcapsules to immunologically relevant molecules an anti-mouse major histocompatibility antigen (MHC class II) [25] was used. Sixty microcapsules with embedded MIN-6 cells were picked randomly. 200 µL of 2% Bovine serum albumin (BSA) (Sigma, UK), and Hanks' Balanced salt solution (HBSS) (Sigma, UK) (blocking buffer) was added to the particles, followed by addition of 2 µL of antibody solution (0.2 mg/mL). The suspension was then incubated at 37℃ for 1h. Capsules were then washed three times with HBSS to remove any excess antibody remaining in the solution. For the control sample, non-encapsulated cells were also exposed to the mouse antibody with the same procedure. Samples were then observed using confocal microscopy.

2.6 Performance of fluidised-bed bioreactor

The fluidisation performance of the bioreactor was quantitatively evaluated by measuring the bed expansion parameter. The pump flow rate was increased until no further change in bed height was noticed. At each flow rate, bed height was marked on the bioreactor column, followed by measuring the indicated points. Expansion of encapsulated acellular microparticles bed height versus different flow rates was then calculated using Equation 1.

At each flow rate, bed height was marked on the bioreactor column, followed by measuring the indicated points with a ruler

$$H/H_0 = (1-\varepsilon_0)/(1-\varepsilon) \qquad \text{(Equation 1)}$$

Where H_0 and ε_0 indicate initial bed height and porosity, and H indicates achieved bed height [26].

2.7 Cellular microcapsules stability in fluidised reactor

Microparticles prepared with the same cell density of $2x10^6$ cells/mL, were cultured in both fluidised-bed bioreactor and cell culture flask, and were randomly selected and observed under a light microscope (n= 200). The number of damaged and/or broken microparticles were noted on the day of encapsulation (day 0) and after 1 and 7 days of culture in both static and fluidised culturing conditions.

2.8 Live/ dead assay

The viability of MIN-6 cells encapsulated in alginate microparticles from both the fluidised bioreactor and culture flask were assessed using a live/dead cytotoxicity kit (Invitrogen, UK). Two-colour fluorescence method involving propidium iodide (PI) (indicator for dead cells, 1 mg/mL), and calcein acetoxymethylester (calcein AM) (indicator for viable cells, 1 mg/mL) were chosen. Briefly, 5 µL of calcein AM and 25 µL of PI was added to 2 mL of capsules suspended in DMEM, followed by incubation at 37℃ for 40 min. After staining, the cells were imaged using confocal laser scanning microscopy (Olympus FV1000, Multiple Ar laser, Germany), and raw data from z-stack was analysed with Imaris software (Bitplane, UK).

2.9 Insulin secretion

To investigate the functionality of encapsulated MIN-6 cells cultured in both the fluidised-bed bioreactor and static condition, the secreted amount of insulin in response to changes in glucose was measured using glucose-stimulated insulin secretion (GSIS) assay. Briefly, microcapsules collected from both groups were washed and pre-incubated in Krebs-Ringer bicarbonate buffer (KRBH: 125 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM $NaHCO_3$, 10 mM HEPES, 1.19 mM KH_2PO_4) + 0.1% BSA for 2 h. Microcapsules were then statically incubated for 1 h with low (2 mM) glucose concentration in KRBH+ 0.1% BSA followed by the addition of high (20 mM) glucose concentration in KRBH+ 0.1% BSA and the stimulator and samples incubated for another hour. For each incubation period, the cultured supernatant was collected during GSIS assay. The supernatant obtained by centrifugation of the dissolved particles was then diluted to the appropriate range based on the assay standard curve. Secreted insulin was measured using a rat/mouse insulin enzyme-linked

immunosorbent assay (ELISA) kit (Millipore, USA) and the colorimetric reaction was quantified using a plate spectrophotometer (GloMax-Multi+Mictoplate Multimode reader, Promega, USA) at a wavelength of 450 nm [27]. Results were normalised by measuring the number of encapsulated cells in each sample using a CyQUANT cell counting kit (Invitrogen, UK), which expresses strong fluorescence after binding to the cellular nucleic acid. The insulin-stimulated index (SI) was calculated from the ratio of insulin secreted under high and low glucose stimulation.

2.10 Statistical analysis

Results are presented as mean ± standard error of the mean unless otherwise stated. Student's t-test, assuming equal variance, was used to identify any significant differences between pairs of groups. A p-value < 0.05 was determined as significant.

3 Results

3.1 Uniform microcapsules

The particles manufactured using the vibrating nozzle and extrusion methods were compared (Figure 1). Optimised acellular alginate particles produced using the vibrating nozzle method exhibited an average diameter of 200 μm and less than 9% relative standard deviation (Figure 1b and c). A comparison of the particle size distributions achieved using both techniques revealed a narrow size range (180 – 220 μm) and high uniformity for particles produced using the vibrating nozzle. While use of the extrusion method resulted in a broader size range from 180-1140 μm (Figure 1d). Due to the narrow size distribution achievable using vibrational nozzle set-up, all further experiments used particles manufactured using this technique. Additionally, z-stack imaging using confocal microscopy allowed 3-dimensional (3D) visualisation of encapsulated MIN-6 cell location within the alginate matrix. Cells were observed to be distributed homogeneously between the core and the inner surface of alginate hydrogels (Figure 2a, b).

3.2 Antibody exclusion

To investigate the permeability of 1%w/v alginate microcapsules to the immunologically relevant molecules, encapsulated cells were exposed to FITC-mouse antibody, which can target the MHC class

II antigens on the surface of pancreatic beta-cells. Compared to the non-encapsulated cell suspension in medium (control) (Figure 2c), encapsulated MIN-6 cells within alginate microcapsules were successfully protected from the antibody. No fluorescence was detectable in the encapsulated samples using confocal microscopy (Figure 2d, e, f, and g).

3.3 Fluidisation of microparticles

Fluidisation of MIN-6 embedded microcapsules within the bioreactor was initiated by the passage of an upward flow of cell culture medium through the fixed bed of accumulated microcapsules located at the bottom of the column (H_0= 1cm). Further increases in fluid flow rate, forced upward movement of alginate particles, followed by the expansion of the fluidised bed until the particles reached the top filter. Quantitative evaluation of bed expansion versus fluid flow rate was determined as an important parameter in evaluating fluidisation quality. For fluid flow rates up to 100 µL/min particles were floating within the column with a cluster-wise movement. Complete suspension of particles within the entire length was achieved at 2000 µL/min (superficial velocity [volumetric flow rate/ cross sectional flow area] = 1.13 cm/min) (Figure 3b).

3.4 Stability of alginate microcapsules

A comparison of cellular microcapsule stability in fluidised versus static culture was investigated at the maximum flow rate of 2000 µL/min. Alginate capsules from both fluidised and static culture were optically observed in search of any ruptured microcapsules (Figure 3c). The percentage of damaged cellular microcapsules from both fluidised and static culture differed by less than 2% on day 1 and 7 (Figure 3d). Overall, among the entire randomly selected microcapsules (n = 200), only six damaged capsules were found in the fluidised set-up on day 7.

Further investigation of cellular particle stiffness showed no significant difference between swelling degree when cultured in fluidised-bed bioreactor versus T-75 culture flask after 1 day (P=0.053) and 7 days (p=0.69) post encapsulation (Figure 3e) (supplemental figure 1).

3.5 Evaluation of cell viability

Besides 2-dimensional micrographs of groups of live/dead fluorescently stained MIN-6 cells in alginate capsules, confocal laser scanning microscopy enabled 3D reconstruction of an individual

microcapsule using z-stack imaging technique (Figure 4a, b, and c). Qualitative observation demonstrated high viability of cells cultivated in both the fluidised bed bioreactor and a standard T-75 culture flask after 1 and 7 days of *in vitro* studies.

3.6 Insulin secretion in response to glucose

The secretion of insulin in response to changes in glucose concentration, which is essential to assess the functionality of encapsulated cells *in vitro*, was evaluated using the GSIS assay. Figures 4d and 4e illustrate that encapsulated MIN-6 cells within 1% alginate showed a significant increase in the amount of secreted insulin when transferred from low to high glucose concentration ($P<0.001$), regardless of the culture condition.

On day 1, encapsulated MIN-6 cells cultivated in the fluidised-bed bioreactor secreted 30.2 ng/mL± 5.1 and 103.1 ng/mL± 14.4 in response to 2 mM and 20 mM glucose concentration, respectively (SI= 3.4). In contrast, cells cultured in static culture secreted 20.7 ng/mL± 3.5 in low and 50.3 ng/mL± 1.9 in high glucose concentrations (SI= 2.4). The amount of insulin secreted by beta-cells at a high glucose concentration was found to be significantly different between the two culture methods ($P= 0.002$) (Figure 4d).

The same trend was observed on day 7 post-encapsulation; MIN-6 microcapsules cultivated in the fluidised bed bioreactor secreted significantly more ($P< 0.001$) insulin when exposed to higher glucose concentration compared to static culture. Samples from the fluidised-bed bioreactor secreted 46.6 ng/mL± 12.3 and 170.7 ng/mL± 13.9 in high and low glucose concentration (SI= 3.7), respectively, while samples from static culture only secreted 32.6 ng/mL± 6.6 in 2 mM glucose solution, and 74.3 ng/mL± 0.2 in response to 20 mM glucose concentration (SI= 2.3) (Figure 4d).

4 Discussion

The value of encapsulatiing living cells in polymeric carriers has long been recognised as enabling the translation of cell-based therapies, particularly for autoimmune diseases such as type I diabetes, since these approaches may camouflage the encapsulated cells from the host's immune system. In this work, pancreatic MIN-6 cells were encapsulated in 1% alginate microparticles utilising a vibrating nozzle technology in which a laminar fluid jet will naturally break into droplets at a particular

frequency, resulting in jet breakup to nearly double the internal diameter of the nozzle [2]. In comparison to conventional extrusion methods that often fail to generate repeatable alginate microparticles with a narrow size distribution, the whole vibrating nozzle encapsulation process developed in this study was simple and quick (~5 min for encapsulating 10 ml of cell suspension in polymer) as well as capable of generating relatively uniform micro-droplets with a well-defined diameter (Figure 1d).

The entire encapsulation process was performed at room temperature under physiological pH. Preliminary studies were conducted to optimise the experimental parameters, such as, polymer flow rate, vibration frequency, polymer concentration, cross-linking solution, and hardening time. These parameters were found to influence the final average diameter and size distribution of the generated particles (supplemental Figure 2). Optimisation of these parameters resulted in the generation of spherical microparticles with an average diameter of 200 µm± 19 when using a 1%w/v alginate solution (Figure 1b, c). The formation of microcapsules with appropriately sized diameters is critical since particles > 300 µm have been associated with oxygen limitations and hypoxia (>300 µm) [7, 8, 28]. Consequently, manufacture of mono-modal cellular microcapsules in this study allowed the potential for application of fluidised-bed bioreactor.

Up to now, a variety of bioreactors have been studied for a wide range of applications, including biopharmaceuticals, bacteria and yeast culture, enzyme production, scaffolds, and mammalian cell culture (mainly for hepatocytes, and bone marrow stromal osteoblasts) [29, 30]. Herein, a simple fluidised-bed bioreactor with a volume of 15 mL was studied to cultivate pancreatic MIN-6 cells encapsulated within alginate. The application of a fluidised-bed for culture of mammalian cells has several advantages compared to other types of bioreactors. This mainly includes their ability to allow sufficient mass transfer, owing to the perfusion of cell culture medium through the bioreactor column, which enables direct contact between the encapsulated cells and the medium for the exchange of nutrients and oxygen [31]. Moreover, the simplistic design of fluidised bioreactors in comparison with other bioreactor types, such as hollow fibres, allow uncomplicated cleaning and sterilisation, which makes these devices even more suitable for scale-up[32].

Mammalian cells lack the cell wall that exists in plant cells, this makes them more vulnerable to shear forces imposed by mechanical mixing in pneumatically, and STR bioreactors. Hence, the application of a fluidised bioreactor, based on the upward flow of the medium, provides a well-mixed liquid that completely removes the need for mechanical mixing with an impeller or aeration, and this improves mammalian cell survival compared to other mentioned bioreactors [18, 33]. Therefore, a fluidised-bed bioreactor as a device with a simple design and low manufacturing cost, not only provides a suitable environment to the cultured cells within the immunoisolated system, but could also overcome the existing transplantation challenge by providing a handling method between manufacturing and clinical site. This approach could also be beneficial to minimising the loss and damage of islets prior to transplantation, especially for cells with insufficient donor supply, such as pancreatic beta-cells obtain from deceased donors [34].

In this study, characterisation of fluidisation quality within the column was evaluated by measuring bed expansion versus medium flow rates (Figure 3b). The value of bed expansion is related to factors such as fluid velocity, micro-carriers diameter, and fixed-bed height (H_0) [35]. Formation of microparticles with high uniformity, using vibrational technology, enhanced homogeneous mixing of particles throughout the entire bioreactor column. This was confirmed by evaluating the viability and insulin secretion sensitivity of encapsulated cells (Figure 4). Further, at maximum flow rate (2000 µL/min) expansion of particles throughout the entire column was achieved with low microcapsules breakage (Figure 3c, d). This result, in parallel no significant difference in the swelling degree of cellular microcapsules cultured in fluidised-bed bioreactor or T-75 flask (supplemental Figure 1), suggests the promising performance of fluidised bioreactor used in here for cultivation of encapsulated MIN-6 cells within 1% alginate microparticles.

The biological activity of MIN-6 cells with and without fluidisation was another important aspect evaluated in this study. The total cell concentration used here was sufficient to evaluate cell survival and their response to changes in glucose concentration using insulin ELISA. However, in vivo studies would contribute more details about the number of required cells to maintain normoglycaemia. The high viability of encapsulated cells cultivated in fluidised conditions was established by confocal laser scanning microscopy, shown in Figure 4a, b, and c. Results suggested that fluidisation achieved in the

bioreactor column did not have any harmful effect on cell viability. Additionally, the determination of MIN-6 cells ability to remain functional in fluidised culture revealed that encapsulated cells cultivated within the bioreactor exhibited higher insulin sensitivity compared to the control group (Figure 4d, e). Notably, comparison of results obtained from GSIS assay on day 1 versus 7, showed the insulin-stimulated index of encapsulated MIN-6 cells in static culture was slightly reduced from 2.4 to 2.3. Whereas, in the case of the fluidised-bed bioreactor, in conjugation with encapsulated cells ability in secreting more insulin on day 7, the value of the insulin-stimulated index also exhibited an increasing trend from 3.4 to 3.7.

Moreover, during 7 days of *in vitro* culture, the fluidised bed was connected to 20 ml of cell culture medium container without the addition of fresh culture medium through the *in vitro* fluidisation, which to a great extent can reduce the risk of contamination owing to fewer requirements for opening the sterile bioreactor circulating loop (Figure 3a). Whereas, in the case of encapsulated cells within the static cell culture flask, the medium needed to be diluted with fresh DMEM every other day to provide essential nutrients, and prolong the microparticles hydration. This, in fact, shows the ease of application of fluidised bioreactor for maintaining encapsulated cells viability and functionality in the clinical sites, even if there are unexpected delays in surgical transplantation, which currently with conventional static cell culturing methods cannot be easily maintained [1].

5 Conclusions

To our knowledge this study demonstrates, for the first time, the application of a fluidised-bed bioreactor for the cultivation of endocrine pancreatic beta-cells compared to static culture. Herein, the development of alginate microcapsules with uniform size and shape by utilising vibrating nozzle technology is reported. This enabled the homogeneous fluidisation of microparticles in a fluidised-bed bioreactor. High viability, enhanced insulin sensitivity to changes in glucose concentration, and minimal damage to microparticle morphology was observed in the fluidised system compared to static culture. These results suggest that cultivation of encapsulated cells within the bioreactor with relatively small dimensions could provide a simple solution for the transfer of encapsulated cells from laboratories to clinical sites, and consequently extend the future of cell-based therapies.

Supporting information

The following files are available free of charge.

Supplemental Figure 1: comparison between swelling degree of cellular alginate microcapsules in fluidised versus static culture.

Supplemental Figure 2: evaluation of processing parameters influences on the average diameter of alginate microparticles produced by vibrating nozzle technology.

Discloser statement

No competing financial interest exist.

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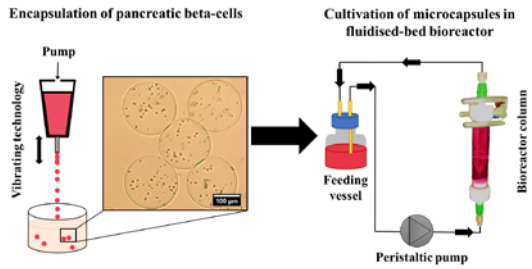
Table of contents graphic (TOC)



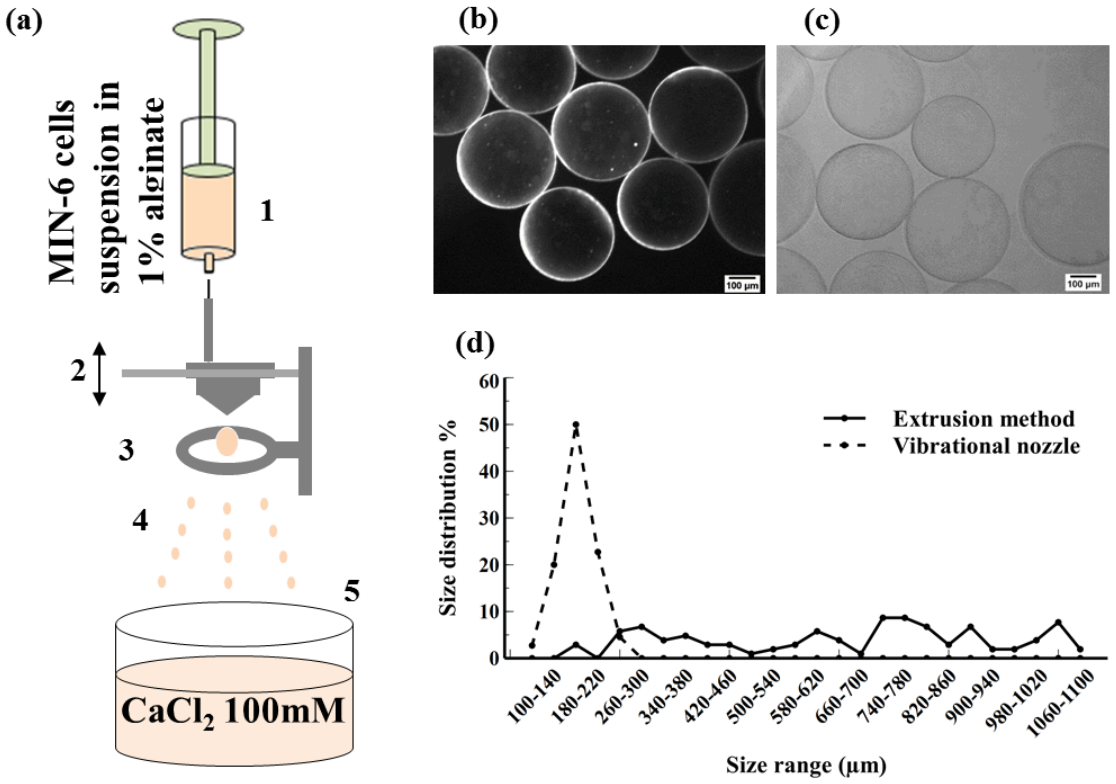
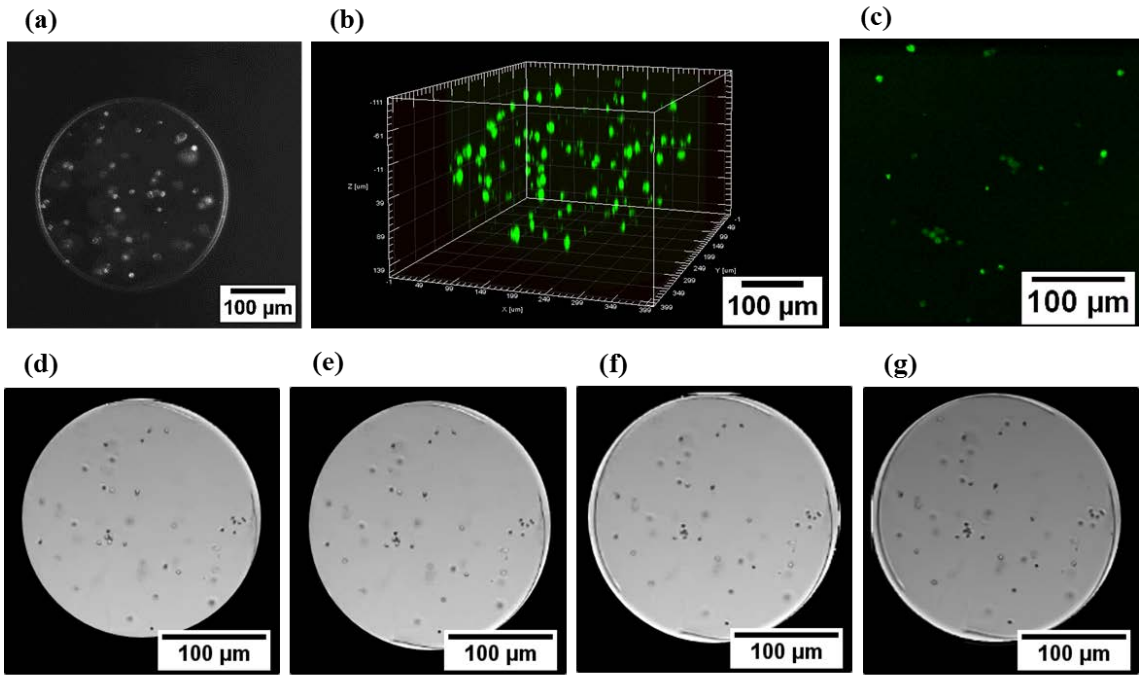
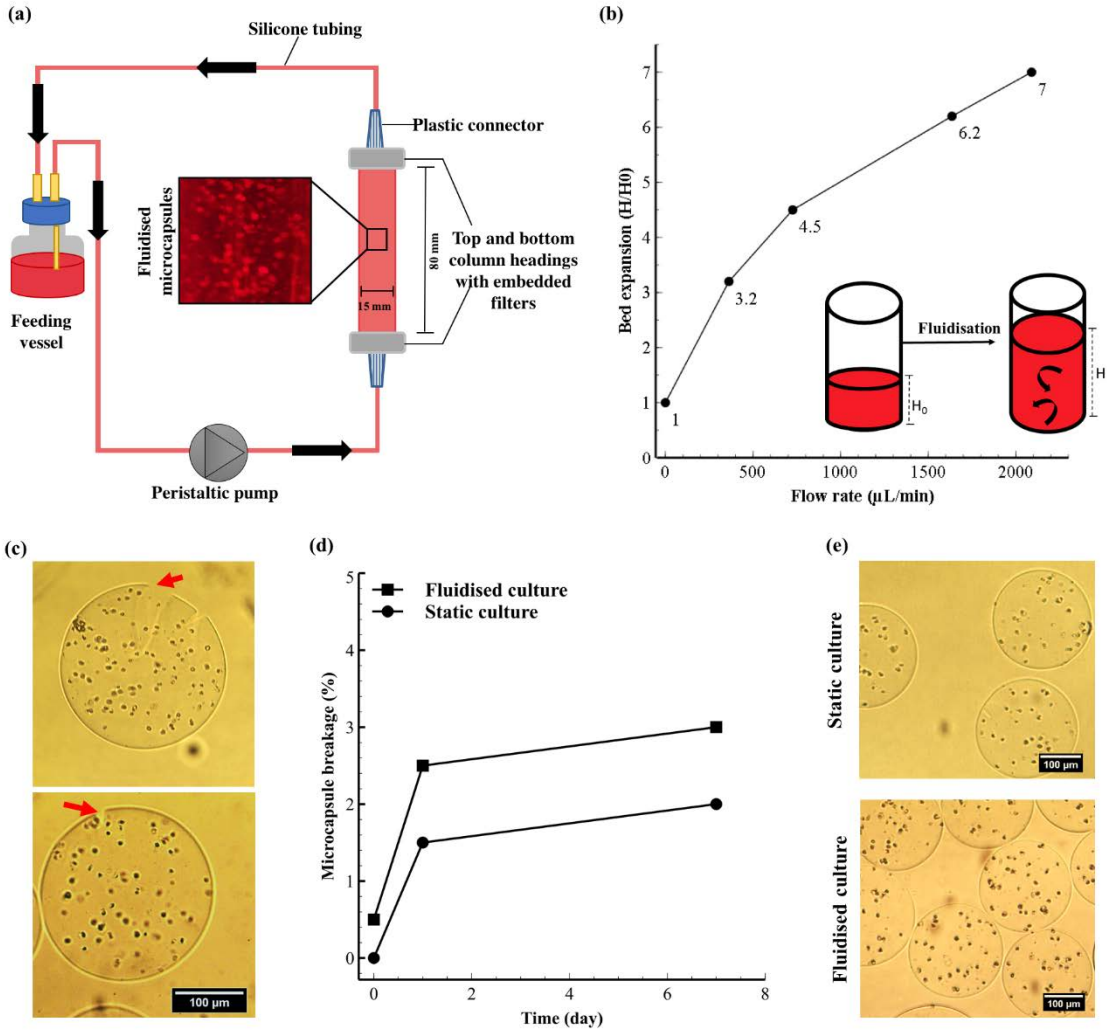


Fig 1. (a) Schematic of alginate microparticle production using vibrating nozzle technology: (1) Pumping of alginate and cell suspension with syringe pump, (2) Mechanical vibration of the nozzle at set frequency, (3) Droplet formation from the nozzle head, (4) Formation of uniform spherical drops due to the controlled break-up of the liquid jet and electrostatic charge, (5) Cross-linking solution. **(b)** and **(c)** Phase contrast and optical micrographs of optimised void alginate microparticles, respectively (frequency=3000 Hz, Alginate conc. =1w/v%, $CaCl_2$ conc. =0.1 M, and pump flow rate=15 ml/min, nozzle size=80 µm). **(d)** Comparison between size distributions of alginate microparticles prepared



with vibrational nozzle and conventional extrusion technique, respectively (n=110 microcapsules). Scale bar = 100 µm.

Fig 2. Evaluation of cell distribution, and immunoisolation within alginate microcapsules. **(a)** Two-dimensional phase contrast micrograph of an individual microparticle with embedded MIN-6 cells; scale bar = 100 µm. **(b)** Three-dimensional structure of the same microparticles using confocal laser scanning microscopy, and Z-stack imaging (stacks of 2-D images, occupying the same x-y position but varying along z-axis). **(c)** Confocal images of non-encapsulated MIN-6 cells (control) after being exposed to FITC-mouse antibody. **(d, e, f,** and **g)** Two channel Z-stack image of cells embedded in



alginate microparticle at 24 µm, 36 µm, 48 µm, and 60 µm from centre of the alginate particle, respectively (the merged images were acquired by transmitted imaging and FITC channel).

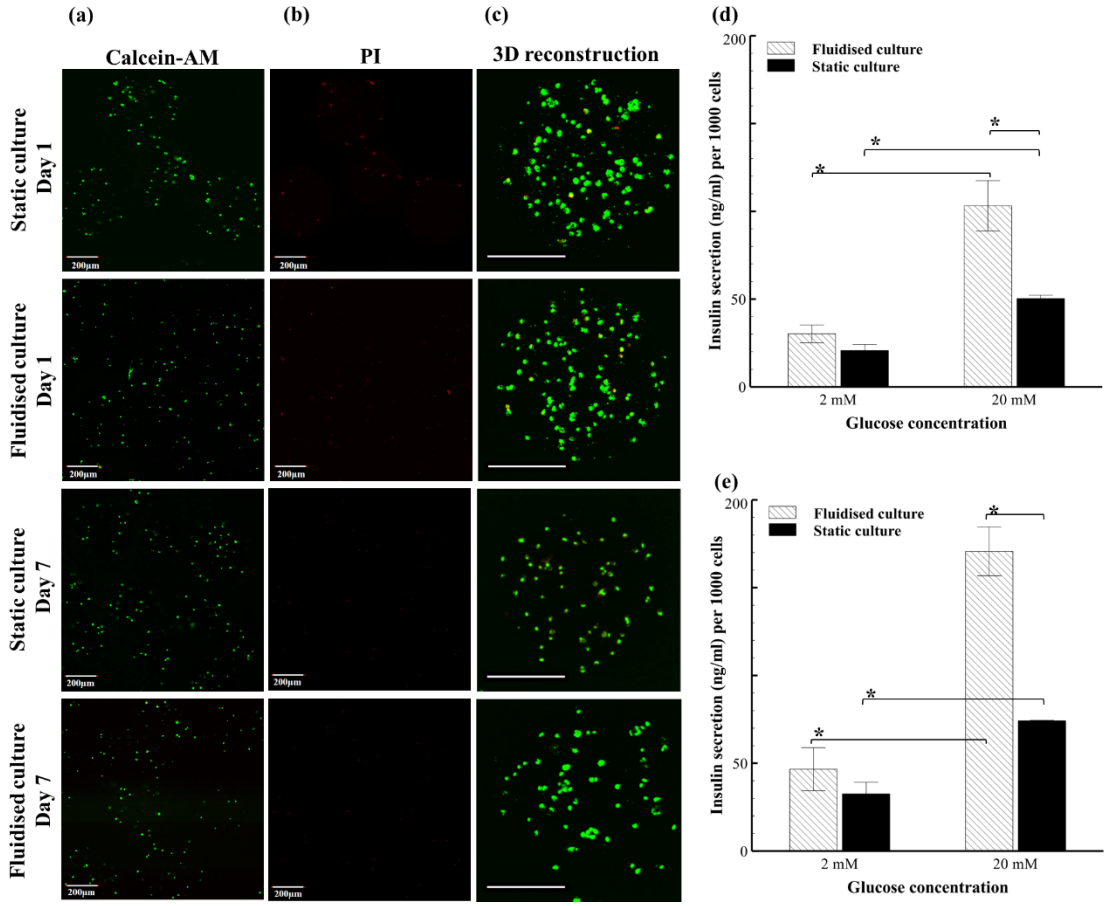


Fig 3. (a) Schematic diagram of fluidised-bed bioreactor for cultivation of encapsulated pancreatic MIN-6 cells. **(b)** Quantitative characterisation of fluidisation quality using bed expansion parameters (H_0 and H indicate initial bed height, and achieved bed height versus flow rate, respectively). **(c)** Light micrograph of damaged alginate microcapsules captured on day 7. Scale bar = 100 μm. **(d)** Comparison of fluidisation impact on cellular alginate microcapsules stability under static and fluidised culture (particles number = 200). **(e)** Light micrographs of MIN-6 pancreatic cells embedded within alginate microcapsules cultured for 7 days in a fluidised-bed bioreactor and T-75 cell culture flask.

Fig 4. Evaluation of encapsulated cell viability and functionality in fluidised versus static culture. **(a)** Unmerged two-dimensional confocal micrographs of live cells (calcein AM marker: green channel) within a group of alginate microcapsules. **(b)** Unmerged two-dimensional confocal micrograph of dead cells (PI marker: red channel) within a group of alginate microcapsules. **(c)** 3-dimensional reconstruction of one cellular alginate microcapsule with merged green and red channels. Insulin secretion of MIN-6 cells in response to glucose stimulation; insulin release was normalised against 1000 cells on **(d)** days 1, and **(e)** day 7, respectively. Results are represented mean ± SEM (n= 4), (*P<0.05). Scale bars = 200 µm.

Supporting Information

"Encapsulation and fluidisation maintains the viability and glucose sensitivity of beta-cells"

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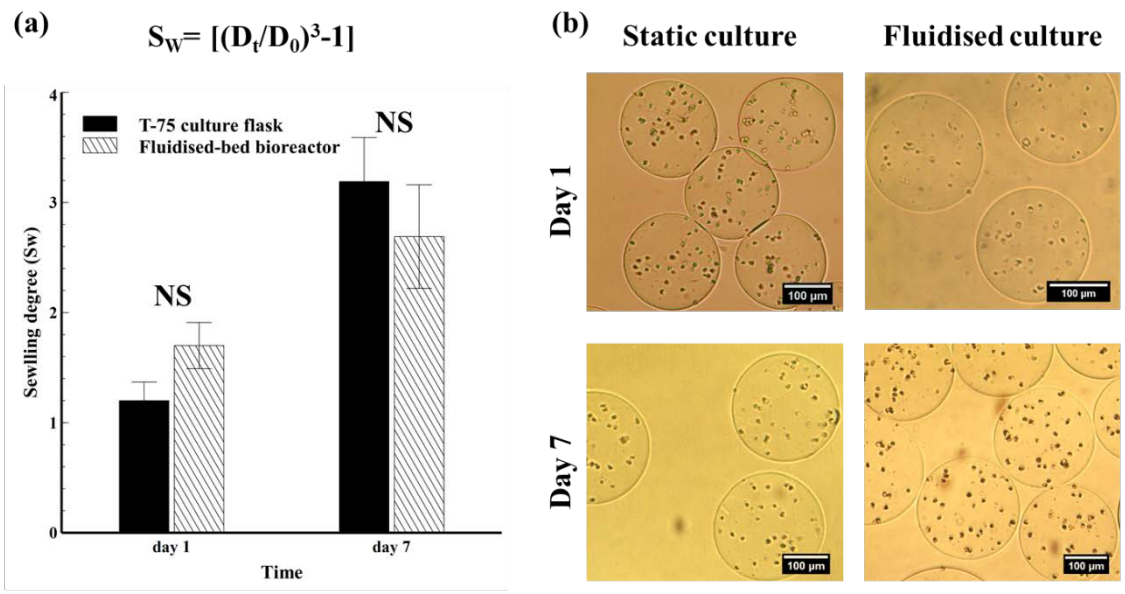
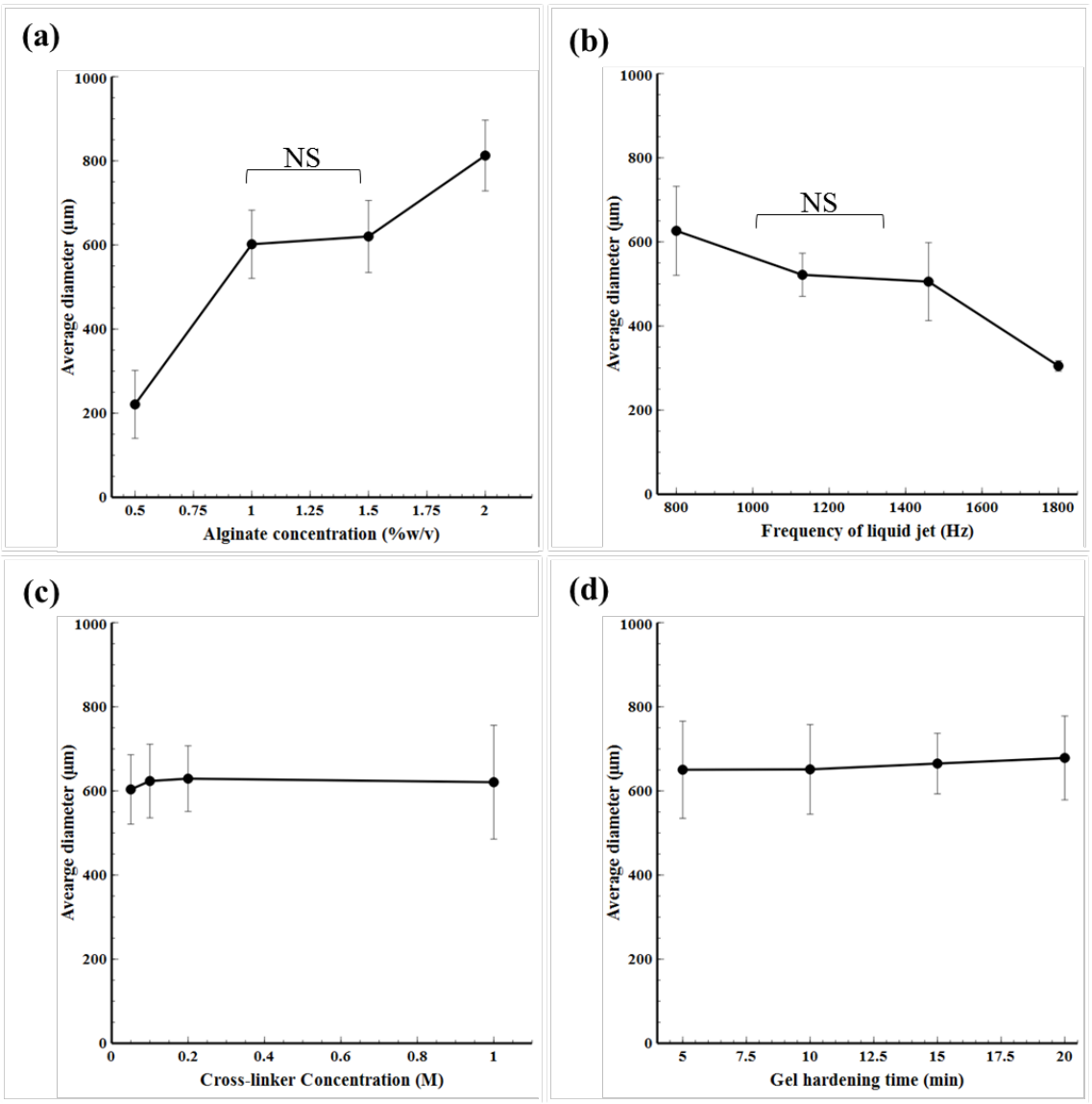


Supplementary fig 1. (a) Comparison of the swelling degree (D_0 indicates average diameters of alginate microparticles on day 0, and, D_t indicates average diameter on day 1or 7) of cultured cellular alginate microcapsules under static and fluidised (flow rate= 2000 µL/min) conditions on days 1 (NS, P=0.053) and 7 (NS, P=0.69), (particles number = 50). **(b)** Optical micrographs of MIN-6 pancreatic cells embedded within alginate microcapsules cultured for 7 days in a fluidised-bed bioreactor and T-75 cell culture flask. Scale bars = 100 µm. NS=non-significant.



Supplemental Figure 2. Effect of individual processing parameters on average diameter and size distribution of alginate microparticles, produced by vibrating nozzle technology (nozzle inner diameter: 120 µm). **(a)** Alginate concentration 0.5-2% w/v ($P<0.01$) (frequency=1000 Hz, $CaCl_2$=0.1 M, hardening time=10 min), **(b)** Frequency of vibrating nozzle 800-1800 Hz ($P<0.01$) (alginate concentration=1%, $CaCl_2$=0.1 M, hardening time=10 min), **(c)** Cross-linked solution concentration 0.05-1 M ($P>0.05$) (alginate concentration=1%, frequency=1000 Hz, hardening time=10 min), **(d)** Gel hardening time 5-20 min ($P>0.05$) (alginate concentration=1%, frequency=1000 Hz, $CaCl_2$=0.1 M). Results are presented as mean± S.D. (number of microparticles = 20). In each experiment, a single processing parameter was varied and all others were constant, NS = non-significant.